Comparison of Calcium-Induced Associations of Bovine and Caprine Caseins and the Relationship of α_{s1}-Casein Content to Colloidal Stabilization: A Thermodynamic Linkage Analysis[1]

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ABSTRACT

In milk, α_{s1}-, α_{s2}-, β-, and κ-caseins undergo association into colloidal complexes (casein micelles) that are visible with the electron microscope. Hydrophobic interactions and Ca^{2+} bonding are among the major causes of colloidal complex formation. In model systems, stable colloidal casein micelles can be obtained in a calcium caseinate solution by centrifugation at $1500 \times g$. The stabilities of colloidal complexes of bovine and two caprine caseins, selected for their α_{s1}-casein contents, were tested and thermodynamically linked with the calcium-induced changes in the amount of stable colloid present. Analysis of the data according to this thermodynamic linkage approach for bovine and caprine caseins indicates colloid formation at low calcium concentrations (<.015 *M*). However, at increased calcium ion concentrations, these colloids are destabilized. Bovine casein (α_{s1}-casein = 38% of total casein) was most stable with respect to added calcium ion concentration, and caprine casein, low in α_{s1}-casein (5% of total casein), was least stable. The high caprine (α_{s1}-casein = 17% of total casein) was intermediate in stability. After destabilization, bovine casein was not resolubilized at elevated calcium concentrations, but both caprine caseins were. More casein from the low α_{s1}-casein sample could be resolubilized (salted in). These results suggest a role for casein composition in dictating the functional properties of milks from various species.

(**Key words**: colloidal stability, calcium binding, casein, thermodynamic linkage analysis)

Abbreviation key: $\mathbf{k_1}$ = colloid destabilization constant; $\mathbf{k_2}$ = salting-in constant.

INTRODUCTION

The caseins are the main protein components of bovine milk and consequently play an important role in the stability of dairy products. Casein occurs in milk primarily as spherical colloidal particles with average diameters of about 150 nm (19). These particles, the casein micelles, theoretically consist of a large number of spherical subunits with average diameters of about 20 nm called submicelles (16, 17). Submicelles, or casein aggregates, survive exhaustive dialysis against distilled water and consist mainly of protein. The association of the casein molecules to form polymeric species depends on the hydrophobic character of the major casein components: α_{s1}-, α_{s2}-, β-, and κ-caseins. The phosphate groups of α_{s1}-, α_{s2}, and β-caseins react with calcium ions to link the submicelles together, either directly or in chains involving inorganic phosphate and citrate. Studies with purified caseins show that individual α_{s1}-, α_{s2}-, and β-caseins are readily precipitated by calcium, but κ-casein will stabilize the α_{s1}-,

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α_{s2}-, and β-casein components against the flocculating action of calcium ions by the formation of stable synthetic micelles (7, 21, 22). Calcium binding to caseins is important not only in the formation of the micelle in vivo but also in food processing when calcium caseinates are used as ingredients.

In any model of the casein micelle, the characteristics of the component caseins must be taken into account, particularly since Schmidt and Koops (18) have shown that, for synthetic micelles, changes in the composition of the casein components can alter micellar functional properties. Although the ratios of the various casein components of bovine milk are relatively constant (6), the composition of caprine caseins may vary, particularly the α_{s1}-casein component (3, 13). This in turn is thought to have an influence on the properties of the caprine milks (1, 5). Thus, comparisons of caseins of known ratios of α_{s1}-casein can afford insights not only into species variation but also into the functionality produced by unique protein-protein interactions. To provide insight into the forces involved in the stabilization of colloidal aggregates of caseins that occur in bovine and caprine milks, the effects of calcium ions on bovine and caprine whole caseins, their stabilization into colloidal complexes, and subsequent colloid stability were investigated. Our data were analyzed by employing previously developed computer-generated models (9, 12) based on concepts of thermodynamic linkage (24). This study is aimed at relating the stability of casein colloidal complexes to the manufacturing characteristics of bovine and caprine milks.

MATERIALS AND METHODS

Deionized Water

Deionized water, prepared by passage of distilled water over a mixed bed cation-anion exchanger, was used throughout the study.

Sources of Caseins

Whole caseins were prepared by isoelectric precipitation of the individual skim milk samples obtained from a Jersey cow and French-Alpine goats. The precipitate was dissolved by addition of NaOH to yield a solution of pH 7.0. The casein was reprecipitated, washed, and then resuspended. The sodium caseinate was subsequently cooled to 4°C and centrifuged at 100,000 × g for 30 min to remove residual fat. Finally, the suspension was dialyzed versus cold deionized water at 4°C for 72 h with three changes and then lyophilized.

The integrity of the samples was confirmed by SDS-PAGE, and the percentages of the various component caseins were estimated by densitometry as previously described (2). The two caprine milks selected for this study were screened by reversed-phase HPLC (13), and their relative casein contents were quantitated by densitometric measurements of their SDS-PAGE patterns (2).

Model Casein Micelles (Colloidal Stability Test)

Caseins (about 20 mg/ml) were dissolved in water, adjusted to pH 7.0 with .1N KOH or NaOH, and equilibrated in a water bath at 24°C for 15 to 20 min. To 2 ml of protein solution (in thick-walled centrifuge tubes), 2 ml of desired calcium chloride in imidazole·HCl at pH 7.0 were added. The tubes were inverted twice and allowed to stand in a 24°C water bath for 30 min.

Tubes were centrifuged at 1500 × g for 10 min. One milliliter of the supernatant was added to a 10-ml volumetric flask containing 1 ml of 1N sodium citrate, plus 1 ml of water, and the mixture was diluted with water. Any turbid solutions were cleared by addition of .1 ml of .1 M Na_2-EDTA, and the supernatant protein concentration was determined at 280 nm. An absorptivity of .850 ml/mg cm at 280 nm was used for whole casein (16).

Theory and Data Analysis

Wyman's (24) concept of linked functions was useful for the treatment of calcium-induced changes in the solubility and the colloidal stability of bovine and caprine caseins (8, 9, 12). The dependence of calcium-induced precipitation (salting out) and resolubilization of purified caseins at higher calcium chloride concentrations (salting in) on temperature and ionic strength gave further insight into the importance of α_{s1}- and β-casein participation in the intermolecular interactions. Historically,

the term "colloid stability" was introduced as a functional test to determine the success of reconstitution of casein colloids reformed from purified components (12, 22, 23). Here, we are applying the theory of thermodynamic linkage to quantify calcium-induced changes in colloidal stability of whole bovine and caprine casein micelles in order to remove the effect of natural variation in the α_{s1}-casein content on colloid stability.

For the biphasic behavior observed (for example, Figure 3), we assumed two classes of binding sites linked to the change in colloid stability. The complete rationale for the following derivation has been given by Kumosinski and Farrell (12). We first assume that the following equilibria occur:

$$\underset{(S_0)}{p} + nx \overset{k_1^n}{\underset{}{\rightleftarrows}} \underset{(S_1)}{PX_n} + mx \overset{k_2^m}{\underset{}{\rightleftarrows}} \underset{(S_2)}{PX_nX_m}, \quad [1]$$

where p is the unbound protein, x is the free salt, n and m are the number of moles of X bound to the two sites, and k_1 and k_2 are the apparent binding constants per site. Here S_0 is the solubility of caseinate before colloid formation (no Ca^{2+}), and S_1 and S_2 are the solubility (colloid stability) of each of the various colloidal species. The mathematical relationship representing this stoichiometry can be represented according to the following:

$$S_{app} = S_0f(p) + S_1f(PX_n) + S_2f(PX_nX_m), \quad [2]$$

where S_{app} is the apparent colloid stability (solubility) at a given total salt concentration (X_T), f(i) are the protein fractional components of species i, and the S are the solubility or colloid stability of each species. For this study, S_1 and S_2 will be relative to S_0. Incorporation of the salt-binding equilibrium constants as defined by Equation [1] into [2] leads to the following:

$$S_{app} = \frac{S_0p}{p + k_1^npx^n} + \frac{S_1k_1^npx^n}{p + k_1^npx^n} + \frac{(S_2 - S_1)k_2^mpx^m}{p + k_2^mpx^m}, \quad [3]$$

where p is the concentration of the unbound protein, and x is the concentration of unbound salt. Cancellation of common terms yields

$$S_{app} = \frac{S_0}{1 + k_1^nx^n} + \frac{S_1k_1^nx^n}{1 + k_1^nx^n} + \frac{(S_2 - S_1)k_2^mx^m}{1 + k_2^mx^m}. \quad [4]$$

This model represents sequential binding, which brings about change in colloidal stability. That is, $k_1 > k_2$, and the n sites become saturated with x prior to significant binding of x to the m sites. Also, for n or m values >1, k_1 and k_2 represent the mean value for each of the n or m binding sites. In reality n or m moles of salt will bind with only one equilibrium constant (K_1), i.e., $K_1 = k_1^n$ and $K_2 = k_2^m$.

The model in Equation [4] was applied in the present study to compare the calcium-induced changes in colloidal stability of bovine and caprine whole caseins. In the case of purified caseins, either free or total calcium can be used, as has been previously discussed (12). In this functional property test (colloid stability), only the total calcium content is considered because that quantity is most readily measured under practical conditions. Although this restriction represents a point of departure from the normal study of binding isotherms, it provides a practical application for this theory while serving as a point of comparison for bovine and caprine caseins. Changes in k, with binding taken into account, can be readily calculated as previously described (12). The data were analyzed using an iterative nonlinear regression program (NLLSQ in BASIC) on a microcomputer that employed the Marquardt algorithm. This program minimizes the standard deviation of the experimental points from the curve, also known as the root mean square deviation (RMSD), where the RMSD is defined as

$$RMSD = SS/(NO - NP + NX) \quad [5]$$

and SS is the sum of the squares of the differences (YC − Y) between the calculated and observed Y values, NO is the number of data

points, NP is the number of parameters, and NX is the number of excluded parameters. The n and m values yielded the minimum root mean square deviation value for the analysis with the minimum error in k_1 and k_2, as previously shown (12).

RESULTS AND DISCUSSION

Calcium-Induced Colloid Stability Profiles of Bovine and Caprine Caseins

Model colloidal complexes have been previously defined by their stability at 1500 × *g* (12, 15, 21). In model systems (12, 21, 23) in which purified caseins have been mixed together, the addition of calcium causes an apparent precipitation followed by formation of a stable colloid and results in an apparent dip in the profile (Figure 1). As more calcium is added, the colloid is destabilized, and precipitation results. This phenomenon has been discussed for purified caseins (21, 22, 23). Figure 1 also demonstrates the effect of ratio of κ- to α_{s1}-caseins on colloid stability. In the present studies involving whole bovine casein, no dip appeared (Figure 2) even at low calcium concentrations (Figure 2 inset). Here the whole caseins had never been separated by urea purification, and so perhaps no changes in protein structure or interactions were necessary to accommodate calcium binding, or no calcium-induced conformational changes were needed to accommodate colloid formation. The idea that intact whole casein may be different from its urea-purified components is supported by the greater sum of secondary structure in whole bovine casein as observed with Raman spectroscopy compared with that calculated from the purified components (4). For bovine casein, as seen in Figure 2, calcium binds to whole casein, producing a stable colloid up to 50 m*M*; then, as an extension of the previously developed theories on colloid stability (12), k_1 of Equation [4] may be thought of as a calcium binding linked to destabilization of that colloid. In turn, k_2 may be appropriately defined as a resolubilization constant (salting in), the linkage of which is perhaps affected by calcium-induced casein-casein interactions.

The influence of the amounts of α_{s1}-component on model micelle stability was compared on bovine and caprine caseins. The two caprine caseins were selected on the basis of their known α_{s1}-casein contents (13). Densitometric analysis of their SDS-PAGE profiles along with the bovine milk casein used in this study are given in Table 1. Thus, in this work, the amount of α_{s1}-casein present ranges from 5

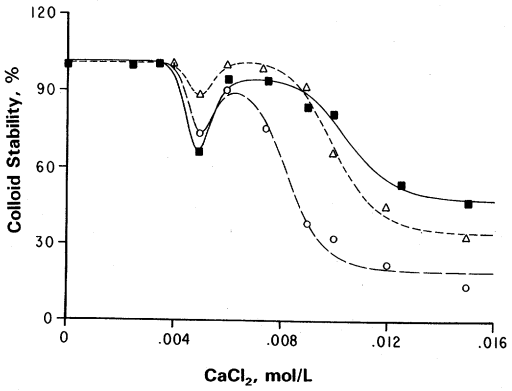

Figure 1. Colloidal stabilities for reformed bovine micelles with varying ratios of α_{s1}- to κ-casein. Supernatant protein at 37°C resulting from the incremental addition of $CaCl_2$ to α_{s1}-casein B plus κ-casein, 40:1 (○); α_{s1}-casein B plus κ-casein, 20:1 (△); and α_{s1}-casein B plus κ-casein, 17:1 (■). Solutions buffered at pH 7.0, 10 m*M* imidazole·HCl. Initial protein = 4 mg/ml. Taken from Kumosinski and Farrell (12).

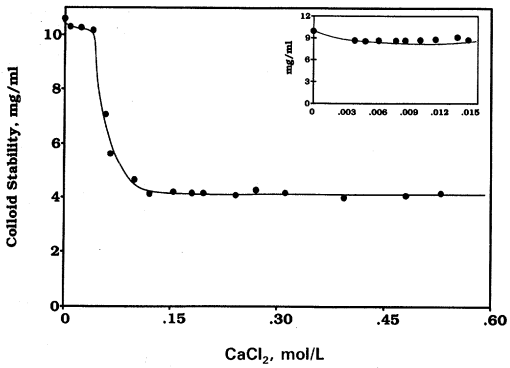

Figure 2. Colloid stability for whole bovine casein. Supernatant protein at 24°C resulting from the incremental addition of $CaCl_2$. Solutions buffered at pH 7.0, 10 m*M* imidazole·HCl. Data are the means of triplicate determinations and fitted with Equation [4]; results are given in Tables 2 and 3. Inset represents an amplification of the data at lower concentrations of calcium ion prior to incipient precipitation. Note that the dip present in Figure 1 is absent.

TABLE 1. Comparison of casein distribution of bovine and caprine caseins by densitometry.

Casein type	Bovine	Caprine[1]	
		Low α_{s1}-casein	High α_{s1}-casein
	(%)		
α_{s2}-Casein	10.0	20.0	6.2
α_{s1}-Casein	38.0	5.0	17.1
β-Casein	40.0	48.0	50.1
κ-Casein	12.0	16.0	17.5

[1]Means of three runs; the casein contents of the low and high α_{s1}-samples were also quantitated by Mora-Gutierrez et al. (13).

to 38% of total casein. The colloidal stability profiles at 24°C and 10 m*M* imidazole·HCl (pH 7.0) of bovine casein were then compared with caprine casein with a low content of α_{s1}-casein and caprine casein with a high content of α_{s1}-casein, as defined in Table 1 (recognizing that bovine casein has decidedly the highest content of α_{s1}-casein).

Earlier studies using moving boundary electrophoresis (11, 14) showed that casein components interact very strongly with each other. The stability of casein micelles is thought to

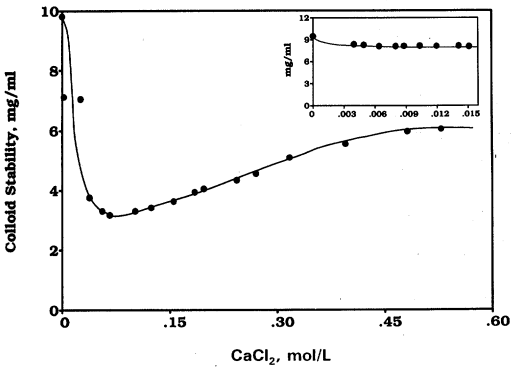

Figure 3. Colloid stability for low α_{s1}-caprine casein. Supernatant protein at 24°C resulting from the incremental addition of $CaCl_2$. Solutions buffered at pH 7.0, 10 m*M* imidazole·HCl. Data are the means of triplicate determinations and were fitted with Equation [4]; results are given in Tables 2 and 3. Inset represents an amplification of the data at lower concentrations of calcium ion prior to incipient precipitation. Note that the dip present in Figure 1 is absent.

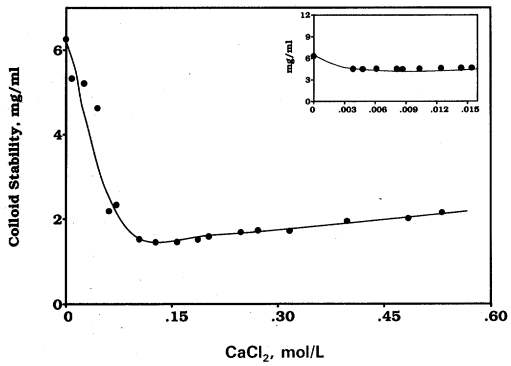

Figure 4. Colloid stability for high α_{s1}-caprine casein. Supernatant protein at 24°C resulting from the incremental addition of $CaCl_2$. Solutions buffered at pH 7.0, 10 m*M* imidazole·HCl. Data are the means of triplicate determinations and were fitted with Equation [4]; results are given in Tables 2 and 3. Inset represents an amplification of the data at lower concentrations of calcium ion prior to incipient precipitation. Note that the dip present in Figure 1 is absent.

evolve from polymerization interactions between α_{s1}-, α_{s2}-, β-, and κ-caseins, moderated by both the ionic environment and temperature (7, 18, 22). The changes in the colloid stability behavior of bovine and caprine caseins in the presence of calcium by centrifugation at 1500 × *g* (Figures 2, 3, and 4) may be intimately related to binding of calcium by the proteins. Addition of Ca^{2+} leads to colloid formation, and stable colloids (defined by 1500 × *g* solubility) occur over a range from 3 to 15 m*M* (inserts of Figures 2, 3, and 4). However, as additional calcium binds to somewhat lower affinity sites, colloid destabilization occurs. For bovine casein the destabilization is gradual, but for the caprine caseins the transition is abrupt, resulting in highly scattered data in the region of incipient precipitation. Analysis of Table 1, using the mean phosphate contents for each casein (7), reveals that the number of phosphates per monomer decreases from 6 for bovine to 4.6 for caprine casein. The high and low caprine caseins are not significantly different in mean phosphate content; α_{s2}-casein appears to compensate for the low α_{s1}-casein content. In both caprine samples, β-casein is the predominant protein. Differences in the colloid stability profiles should relate to changes in protein-protein interactions that

TABLE 2. Comparison of the effects of α_{s1}-casein content on colloid destabilization of bovine and caprine caseins at 24°C.[1]

Whole casein	k_1[2]		n[3]	S_1[4]	
	(L/mol)			(mg/ml)	
	$\bar{X}$	SE		$\bar{X}$	SE
Bovine	17	.1	8	4.1	.1
Caprine					
Low α_{s1}-casein	40	.5	5	3.2	.1
High α_{s1}-casein	22	.1	7	1.5	.1

[1]Solutions buffered at pH 7.0, 10 m*M* imidazole·HCl.

[2]k_1 = Colloid destabilization constant.

[3]Integer values related to cooperativity or to the apparent number of binding sites.

[4]Solubility.

were due to variation in component caseins. This relationship is quantitated by Equation [4] and given in Table 2. Here the colloid stability is highest (lower k_1) for bovine casein, and colloid stability is lowest (highest k_1) for the low caprine α_{s1}-casein. The apparent number of bound calcium ions (n) is greatest for bovine and least for the low caprine α_{s1}-casein, although, in the latter case, both the value of n = 5 approximates the apparent number of moles of phosphate per monomer. In any case, the caprine caseins are much more sensitive to calcium destabilization.

Another interesting difference in behavior was observed between the bovine and caprine caseins. For bovine casein (Figure 2), increased Ca^{2+} does not lead to resolubilization of protein (salting in), which may be attributed to the higher degree of α_{s1}-casein in the bovine protein. In fact, α_{s1}-casein is the most calcium-sensitive protein among the caseins (7, 22, 23) and is the most calcium-insoluble protein. Hence, no k_2 can be calculated (Table 3). The salting-in constants, k_2, are similar for caprine with high content of the α_{s1}-component, compared with caprine casein with low content of the α_{s1}-casein component (Table 3). However, the amount of salting in is significantly different for the low α_{s1}-casein (Table 3), as reflected by a four-fold greater S_2 (the predicted maximum amount of protein salted-in at elevated calcium concentrations). Here the concentration of calcium is given in terms of total Ca^{2+}. This result has a very practical

TABLE 3. Comparison of the effects of α_{s1}-casein content on resolubilization (salting in) of bovine and caprine caseins at 24°C.[1]

Whole casein	k_2[2]		m[3]	S_2[4]	
	(L/mol)			(mg/ml)	
	$\bar{X}$	SE		$\bar{X}$	SE
Bovine[5]	. . .	. . .	. . .	. . .	. . .
Caprine					
Low α_{s1}-casein	3.3	.3	3	3.7	.3
High α_{s1}-casein	2.1	.2	3	1.0	.3

[1]Solutions buffered at pH 7.0, 10 m*M* imidazole·HCl.

[2]k_2 = Salting-in constant.

[3]Integer values related to cooperativity or to the apparent number of binding sites.

[4]Solubility.

[5]No detectable resolubilization (salting in) occurred.

implication because alterations of total calcium under processing conditions can markedly affect products (7, 18).

CONCLUSIONS

Discussion of these results can also be reported in terms of the reciprocal values of k_1 and k_2 (Table 4). If $1/k_1$ is taken as the concentration at which one-half destabilization occurs, then bovine caseins are readily seen to be most stable with increased total calcium content. Because the mean phosphate contents are similar, the amount of calcium binding should be somewhat similar in each case. In contrast, the least stable is the caprine casein with the low α_{s1}-casein content, which is half destabilized at 25 m*M* calcium content. Caprine milk with a low content of α_{s1}-casein has weaker resistance to heat treatments and shorter coagulation times than milk with a high content of α_{s1}-casein (1, 5). The lower stability of caprine micelles with low content of α_{s1}-casein, as noted, may be inferred from the low $1/k_1$ value (Table 4). Several theories of protein solubility (8, 9) have shown evidence that proteins with low charge densities tend to have significantly higher proportions of apolar amino acids on their surface, which would favor hydrophobically driven aggregation and, thus, the resulting insolubility for casein complexes lower in α_{s1}-casein contents and richer in β-casein. The differences in salting in (resolubilization) that were observed between the bovine and caprine caseins (right side of Figures 2, 3, and 4) may be attributed to a number of factors, including differences in the proportion of apolar and polar groups on the surface of these proteins or the size and shape of the casein proteins, resulting from altered casein composition and subsequent protein-protein interactions. With respect to salting in, bovine casein is poorest (none), and the low caprine α_{s1}-casein is greatest. This result is reflected in the highest S_2 (Table 3) and the lower value of $1/k_2$, the half resolubilization point (Table 4). Indeed, differences in α_{s1}-casein content significantly affect colloidal stability and, consequently, the manufacturing characteristics of caprine milk; the high caprine α_{s1}-casein is closer but not identical to bovine α_{s1}-casein in its properties.

Because the reactions of complexes of caseins are related to α_{s1}-casein content, there is a strong indication that the observed differences in physical and chemical characteristics of milks of other species (10, 20) could most likely be explained on a similar basis. Future research in breeding and genetic engineering aimed at alteration of casein content of bovine and caprine milks must take this factor into account.

TABLE 4. Comparison of the reciprocal values of k_1 and k_2.[1]

Whole casein	$1/k_1$	$1/k_2$
	(m*M*)	
Bovine	59	. . .[2]
Caprine		
Low α_{s1}-casein	25	300
High α_{s1}-casein	45	480

[1]These values represent the concentration in m*M* of total calcium at which one-half of the colloid destabilization (k_1) or salting in (k_2) occurs.

[2]No apparent salting in, presumably >500 m*M*.

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